Susceptibility of Sixteen Citrus Genotypes to ‘*Candidatus Liberibacter asiaticus*’

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Abstract

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Huanglongbing (HLB) disease is the most serious threat to citrus production worldwide and, in the last decade, has devastated the Florida citrus industry. In the United States, HLB is associated with the phloem-limited α -proteobacterium ‘*Candidatus Liberibacter asiaticus*’ and its insect vector, the Asian citrus psyllid (ACP; *Diaphorina citri*). Significant effort is being put forth to develop novel citrus germplasm that has a lower propensity to succumb to HLB than do currently available varieties. Effective methods of screening citrus germplasm for susceptibility to HLB are essential. In this study, we exposed small, grafted trees of 16 citrus types to free-ranging

ACP vectors and ‘*Ca. L. asiaticus*’ inoculum in the greenhouse. During 45 weeks of exposure to ACP, the cumulative incidence of ‘*Ca. L. asiaticus*’ infection was 70%. Trees of *Citrus macrophylla* and *C. medica* were most susceptible to ‘*Ca. L. asiaticus*’, with 100% infection by the end of the test period in three trials, while the complex genetic hybrids ‘US 1-4-59’ and ‘Fallglo’ consistently were least susceptible, with approximately 30% infection. Results obtained in this greenhouse experiment showed good agreement with trends observed in the orchard, supporting the validity of our approach for screening citrus germplasm for susceptibility to HLB.

Huanglongbing (HLB), also known as citrus greening, is considered to be the most destructive of all citrus diseases and poses a serious threat to citrus production worldwide (Bové 2006). Although HLB has been known in Asia for over 100 years, the disease first appeared in the western hemisphere only in the last decade, first in Brazil in 2004 (Coletta-Filho et al. 2004), followed by Florida in 2005 (Halbert 2005). HLB was subsequently confirmed in Texas (Kunta et al. 2012), California (Kumagai et al. 2013), and in several countries in the Caribbean basin (Luis et al. 2009). The Florida citrus industry has been especially devastated by HLB. It is currently estimated that 100% of all citrus orchards in Florida are affected by HLB (Spren et al. 2014). As a consequence of HLB, citrus production in Florida has decreased dramatically, with the 2014–15 crop being the smallest in over 50 years (USDA NASS 2015).

The impact, etiology, and management strategies for HLB have been reviewed extensively (Belasque et al. 2010; Bové 2006; Bové and Ayres 2007; Gottwald 2007, 2010; Halbert and Manjunath 2004; Hall and Gottwald 2011). Common initial symptoms of HLB expressed by mature trees are chlorosis in the terminal growth of shoots that may resemble nutrient deficiency, or a characteristic and diagnostic “blotchy mottle” which appears in older leaves located toward the interior of the canopy. Subsequently, canopy thinning becomes apparent, fruit size is reduced, and asymmetrical fruit are frequent. As the severity of HLB progresses, preharvest fruit drop increases, leading to significant loss of yield (Bassanezi et al. 2011, USDA NASS 2015). Fruit harvested from HLB-affected trees typically have reduced soluble solids content and unacceptable flavor compared with those from nonaffected trees (Baldwin et al. 2010; Bassanezi et al. 2009).

HLB is associated with three species of yet-to-be-cultured, phloem-limited, α -proteobacteria: ‘*Candidatus Liberibacter asiaticus*’ in Asia and the western hemisphere, ‘*Ca. L. africanus*’ in Africa and the Middle East, and ‘*Ca. L. americanus*’ in Brazil (Bové 2006). In the orchard, ‘*Ca. L. asiaticus*’ is transmitted from tree to tree by its

insect vector, the Asian citrus psyllid (ACP; *Diaphorina citri* Kuwayama (Hemiptera: Liviidae)). Following infection with ‘*Ca. L. asiaticus*’, depending on the age of the tree at infection, a period of months to years may pass before HLB disease symptoms become visible. However, infected but asymptomatic trees can serve as sources of inoculum (Albrecht et al. 2014; Irey et al. 2006), contributing to the difficulty of managing the disease. As HLB disease progresses, trees become increasingly debilitated and eventually economically nonviable due to low yields coupled with poor fruit and juice quality (Gottwald 2010).

Ultimately, the most effective means to reduce the impact of HLB is citrus varieties with no or a low propensity to succumb to the disease. It is generally recognized that there is no true resistance to ‘*Ca. L. asiaticus*’ infection, and subsequent HLB decline occurs among all of the economically important cultivated citrus types, including sweet orange, grapefruit, lemon, lime, pummelo, and mandarin (Bové 2006; Gottwald 2010). Citrus is a clonally propagated crop, so the chance of identifying meaningful HLB resistance among common commercial citrus cultivars seems extremely remote. Although most cultivated citrus types exhibit the array of symptoms associated with HLB, some types seem less likely to succumb to infection than do others (Folimonova et al. 2009; Shokrollah et al. 2009; Stover and McCollum 2011). Trifoliate orange (*Poncirus trifoliata* (L.) Raf.) is a close relative of *Citrus* spp. and, when compared with standard citrus scion types, *P. trifoliata* had a lower incidence of ‘*Ca. L. asiaticus*’ infection, lower bacterial titer in the plants that did become infected, and less pronounced HLB symptoms (Albrecht and Bowman 2012; Folimonova et al. 2009).

P. trifoliata is sexually compatible with *Citrus* spp. and has been used as a parent in citrus breeding since the end of the 19th century (Swingle 1910). *P. trifoliata* hybrids have become important rootstocks for citrus because *P. trifoliata* imparts traits such as cold hardiness and resistance to *Citrus tristeza virus* as well as resistance to some soilborne pathogens (Soost and Roose 1996).

Due to the reported tolerance to ‘*Ca. L. asiaticus*’ in *P. trifoliata* (Albrecht and Bowman 2011, 2012; Albrecht et al. 2012), there is increased interest in development of complex *Citrus* $\times$ *P. trifoliata* hybrids with enhanced ‘*Ca. L. asiaticus*’ tolerance along with commercially acceptable fruit quality (Stover et al. 2015). In addition to conventional breeding, considerable effort is being put forth to develop HLB-resistant varieties through genetic transformation (Felipe et al. 2013; Grosser et al. 2007, 2011) and hybridization (Albrecht and Bowman 2012; Albrecht et al. 2012). Whether the approach is conventional hybridization or genetic transformation, screening novel germplasm

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for HLB resistance requires evaluation of hundreds to thousands of individuals to find the very few with potential for further evaluation. A major unknown in this process is how best to screen germplasm for resistance to HLB.

Recent work describing evaluation of resistance or tolerance to infection of inoculated trees has been conducted by inoculating with '*Ca. L. asiaticus*' via grafting (Albrecht and Bowman 2012; Folimonova et al. 2009; Shokrollah et al. 2009). However, although grafting can be used for inoculation, grafting is not the means by which infection occurs in the orchard. In addition, when trees are inoculated by grafting, the inoculum load is likely much greater than that introduced by ACP and occurs at a single point (the graft site) rather than at multiple sites, as is the case with ACP.

Another approach used to screen citrus germplasm for resistance to HLB is to establish plants in the field under disease epidemic conditions and rely on resident ACP populations for '*Ca. L. asiaticus*' transmission (Albrecht and Bowman 2011; Albrecht et al. 2014; Shokrollah et al. 2011). However, in orchard trials, successful evaluation is dependent on noncontrolled conditions, because transmission of '*Ca. L. asiaticus*' is by native ACP populations and presumes adequate populations of the vector and that the inoculum are in the vicinity of the experimental site (Albrecht et al. 2014). In addition, young citrus trees growing in the field are susceptible to a variety of natural maladies and environmental changes that can lead to loss for reasons other than HLB, thereby eliminating trees that may have been HLB resistant.

We were interested in determining whether significant differences in the incidence of infection and subsequent '*Ca. L. asiaticus*' titer could be detected among citrus genotypes infected via ACP. We hypothesized that holding small, grafted trees in a greenhouse containing free-ranging ACP populations to transmit '*Ca. L. asiaticus*', along with infected citrus trees as sources of inoculum, could serve as a means to screen for incidence of infection and subsequent bacterial proliferation. To test this hypothesis, we conducted an experiment to determine (i) the kinetics of '*Ca. L. asiaticus*' transmission to citrus by free-ranging ACP in the greenhouse, (ii) whether the progress of infection in the greenhouse is similar to that seen in the field, and (iii) whether significant differences in susceptibility to '*Ca. L. asiaticus*' could be detected among 16 citrus genotypes.

Materials and Methods

Plant material. All citrus plants used in the experiments were produced by grafting '*Ca. L. asiaticus*'-free scion budwood (obtained from the Florida Department of Agriculture and Consumer Services Budwood Bureau, Chiefland) onto seedlings of 'US-812' (Bowman and Rouse 2006) rootstocks. Plants were produced in an ACP-exclusionary

greenhouse at the United States Horticultural Research Laboratory, Ft. Pierce, FL.

Sixteen citrus scion types were included in the experiment (Table 1). Each scion type can be placed into one of five groups: (i) the three progenitors of all commercial citrus types: citron (*Citrus medica* L.), pummelo (*C. grandis*), and mandarin (*C. reticulata*); (ii) the industry-wide standards sweet orange (*C. sinensis*) 'Valencia', grapefruit (*C. paradisi*) 'Marsh', and tangor (believed to be a natural *C. sinensis* × *C. reticulata*) 'Temple'; (iii) complex citrus hybrids ('Orlando', 'Minneola', 'Ambersweet', 'Sunburst', 'Triumph', 'Fallglo', and 'Bower') that have shown varying degrees of susceptibility to HLB in the field in Florida (Stover and McCollum 2011); (iv) *P. trifoliata* × *Citrus* hybrids 'US 119' and 'US 1-4-59', which were included because some reports indicate that *P. trifoliata* and *P. trifoliata* hybrids show a lesser incidence of '*Ca. L. asiaticus*' infection and a lower bacterial titer in infected trees than do members of the genus *Citrus* (Albrecht and Bowman 2012); and (v) *C. macrophylla*, a citrus type reportedly highly susceptible to '*Ca. L. asiaticus*' infection (Folimonova et al. 2009).

For exposure to ACP, trees were placed in a greenhouse owned and operated by the United States Sugar Corporation's Southern Gardens Citrus division in Clewiston, FL. Temperatures in the greenhouse ranged from a high of 35°C during the day in the summer to a low of 15°C in the night during the winter. In addition to the experimental plants, the greenhouse contained free-ranging ACP and numerous trees of varying citrus genotypes infected with '*Ca. L. asiaticus*'.

The experimental design was a completely randomized block with four three-plant replicates of each genotype (3 × 16 × 4 = 192 plants per trial, 576 plants total). The experiment was conducted three times, with starting dates of 30 August 2010, 30 September 2010, and 20 June 2011 for trials 1, 2, and 3, respectively. Plant ages at the time of transfer to the greenhouse containing ACP were 1.5, 2.5, and 9 months for trials 1, 2, and 3, respectively.

Sample collection and assay. Leaf samples were collected prior to transfer of experimental plants into the ACP-inclusionary greenhouse and at regular intervals thereafter for approximately 300 days. The most recent fully expanded leaves were selected for sampling. In trial 1, samples were collected at 16, 31, 50, 78, 109, 157, 199, 247, and 295 days after exposure to ACP. In trial 2, samples were collected at 21, 49, 80, 128, 170, 218, 266, and 337 days after exposure to ACP and, in trial 3, samples were collected at 75, 131, 186, 237, and 312 days after exposure to ACP. The number of sampling times were reduced in the third experiment based on the results obtained in the first two experiments, showing that no '*Ca. L. asiaticus*' was detected until approximately 2.5 months following initial exposure to ACP.

Table 1. Citrus types and cultivars included in the study

Citrus type	Genotype	Cultivar
Citron	<i>Citrus medica</i>	S1
Pummelo	<i>C. maxima</i>	Hirado Buntan
Mandarin	<i>C. reticulata</i>	Dancy
Grapefruit	<i>C. paradisi</i>	Marsh
Sweet orange	<i>C. sinensis</i>	Valencia
Tangor	<i>C. reticulata</i> × <i>C. sinensis</i>	Temple
Tangelo	<i>C. reticulata</i> × <i>C. paradisi</i>	Orlando
Tangelo	<i>C. reticulata</i> × <i>C. paradisi</i>	Minneola
Complex hybrid	<i>C. reticulata</i> × Orlando tangelo [(<i>C. reticulata</i> × <i>C. paradisi</i>) × <i>C. sinensis</i>]	Ambersweet
Complex hybrid	Robinson [<i>C. reticulata</i> × (<i>C. paradisi</i> × <i>C. reticulata</i>)] × Osceola [<i>C. reticulata</i> × (<i>C. paradisi</i> × <i>C. reticulata</i>)]	Sunburst
Complex hybrid	Bower <i>C. reticulata</i> × Orlando tangelo (<i>C. reticulata</i> × <i>C. paradisi</i>) × Temple tangor (<i>C. reticulata</i> × <i>C. sinensis</i>)	Fallglo
Complex hybrid	<i>C. reticulata</i> × Orlando tangelo (<i>C. paradisi</i> × <i>C. reticulata</i>)	Bower
Orangelo (?)	<i>C. sinensis</i> × <i>C. paradisi</i>	Triumph
Hybrid	× <i>Citroncirus</i> spp. (<i>C. paradisi</i> × <i>Poncirus trifoliata</i>) × Ambersweet [<i>C. reticulata</i> × Orlando tangelo (<i>C. reticulata</i> × <i>C. paradisi</i>) × <i>C. sinensis</i>]	US 119
Hybrid	US 119 (<i>C. paradisi</i> × <i>P. trifoliata</i>) × Ambersweet [<i>C. reticulata</i> × Orlando tangelo (<i>C. reticulata</i> × <i>C. paradisi</i>) × <i>C. sinensis</i>]	US 1-4-59
Alemow	<i>C. macrophylla</i>	Not available

Petioles or midribs were collected from a single leaf from each of the three trees comprising a replicate and pooled. The pooled petioles were ground to powder under liquid N. Total nucleic acid was extracted from 100 mg of the powdered petiole tissue using a Qiagen DNeasy kit according to manufacturer's protocol. Nucleic acid was quantified by spectrophotometry (Nanodrop; ThermoScientific).

Nucleic acid extracts were assayed for the '*Ca. L. asiaticus*' 16S ribosomal DNA (rDNA) gene sequence following a standard quantitative polymerase chain reaction (qPCR) protocol (Li et al. 2006). Each PCR was conducted using 100 ng of total nucleic acid as template. Cycle threshold (Ct) values were converted to log copy number of 16S rDNA gene sequences based on a standard curve developed in our laboratory ($\log \text{ copy number [CN]} = 11.5 - [0.3 \times \text{Ct}]$) (McCollum et al. 2014). Ct values of 38.3 (CN = 1) or less were considered to be '*Ca. L. asiaticus*' positive. '*Ca. L. asiaticus*' titers are expressed as number of target copies per 100 ng of total nucleic acid (the amount of template added per PCR).

Statistical analysis. The experimental design was a randomized complete block with repeated measures on the same block at different temporal scales. Population means for '*Ca. L. asiaticus*' incidence (percent total number plants positive) and titer ('*Ca. L. asiaticus*' genomes per 100 mg of tissue equivalent) were calculated for each sampling time.

The severity of infection was calculated by dividing the logCN with maximum logCN obtained from the experiment. The highest '*Ca. L. asiaticus*' titer found in the experiment (5.8×10^5 copies/mg of tissue equivalent) is considered as 100% HLB severity for that sample. Therefore, the ratio and severity of HLB were restricted and scaled between 0 and 1 and considered as a pseudo-binomial dependent variable. A generalized linear mixed model (GLMM) is becoming more common in many disciplines and may be preferred over analysis of variance (ANOVA) for nonnormally distributed data (Berridge and Crouchley 2011). The 'lme4' package (Bates et al. 2014) in the R program (R Development Core Team 2013) was used to fit the GLMM with a logit link function and a binomial error distribution for each "cultivar–elapsed or exposure days" combination. Cultivar and exposure days are modeled as fixed effects and experimental trial (e.g., group trees) as a random effect. Random effects are assumed to be uncorrelated among themselves and uncorrelated with residual effects. Standardized residual and normality plots were used to determine the accuracy of model fit. Various transformations were applied to the dependent variable but none improved the overall accuracy of model fit compared with variable without transformation. The interaction between the two factors (i.e., cultivar–exposure days) was not significant; thus, this term was ultimately excluded from the model. For model simplification, a stepwise posteriori procedure was used to

Fig. 1. Incidence of '*Candidatus Liberibacter asiaticus*' infection in citrus trees grown in a greenhouse containing free-ranging Asian citrus psyllids and citrus infected with '*Ca. L. asiaticus*'. Incidence of infection equals (number of trees with copy number greater than or equal to 1/total number of trees) $\times$ 100. Each data point is the mean of three experiments ($n = 192$); vertical lines through data points indicate standard deviation.

aggregate cultivar with most similar effect. Using ANOVA comparison with a χ^2 test, the simpler model is accepted or justified if there are no significant changes for fitting. We repeated this process until all possible cultivars were aggregated. Then, the difference between aggregated cultivar groups was analyzed using GLMM, with each cultivar group modeled separately as a baseline level for contrast to generate the multicomparison table. The 'lme4' package in R was used to fit GLMM with a logit link function and a binomial error distribution for each cultivar–elapsed or exposure days combination. Cultivar and exposure days are modeled as fixed effects, and experimental trial (e.g., group trees) as a random effect. Random effects are assumed to be uncorrelated among themselves and uncorrelated with residual effects.

Results

Incidence of '*Ca. L. asiaticus*' infection for the total population of experimental plants (trials 1, 2, and 3) steadily increased in a nonlinear manner over the duration of exposure to ACP (Fig. 1.). '*Ca. L. asiaticus*'-positive plants were first detected following 11 weeks of exposure to ACP and inoculum sources. The incidence of infection over time for the total population of experimental plants was similar in each of the three experiments. Following 45 weeks of exposure to free-ranging ACP, approximately 70% of the total population of experimental plants was infected.

As with incidence of infection, '*Ca. L. asiaticus*' titer in the total population of experimental plants increased over time (Fig. 2). In trials 1 and 2, mean titers remained at fewer than 10^1 copies of 16S rDNA gene sequence per milligram of tissue until approximately 200 days after placing the test trees into the greenhouse; whereas, by 200 days, the mean titer in trial 3 exceeded 10^2 copies. The final mean '*Ca. L. asiaticus*' titer in trial 3 was approximately 10^5 copies whereas, in trials 1 and 2, the final titer was closer to 10^3 copies. This difference in final titer could have been a function of tree size because plants in trials 1 and 2 were grown in 0.15-liter pots whereas plants in trial 3 were grown in 3.8-liter pots. Differences in final titers could have also been affected by the total number of ACP in the greenhouse and by the percentage of the ACP population that was carrying and competent to transmit the pathogen.

Because conditions for ACP transmission in the greenhouse do not exactly replicate conditions in managed orchards, we compared the

Fig. 2. Changes in '*Candidatus Liberibacter asiaticus*' titer (log copy number [CN] '*Ca. L. asiaticus*' 16S ribosomal DNA per gram of petiole) as a function of days of exposure to free-ranging Asian citrus psyllids in the greenhouse. Samples were assayed for '*Ca. L. asiaticus*' and cycle threshold values converted to logCN based on a standard curve of $\log \text{CN} = 11.5 - (0.3 \times \text{Ct})$.

distribution of the titers of '*Ca. L. asiaticus*' from our greenhouse study with those from diagnostic samples collected in Florida from trees in commercial orchards. The '*Ca. L. asiaticus*' titers in HLB diagnostic samples collected by scouts in the orchard were most frequently in the range of 10^4 to 10^5 copies/g of tissue (Fig. 3). The most frequent titer found in greenhouse plants was approximately 10^4 copies/mg of tissue and very few samples had a titer greater than 10^4 copies. The numbers of samples with 10^2 , 10^3 , or 10^4 copies were two- to fourfold higher in the greenhouse study. Of note is that diagnostic field samples typically are symptomatic mature leaves whereas, in the greenhouse study, no leaf samples showed recognizable HLB symptoms and the leaves were of varying degrees of maturity.

The frequency distribution of '*Ca. L. asiaticus*' titers at 11, 17, 24, 31, and 45 weeks of exposure to ACP was evaluated. The analysis revealed that, over time, a bimodal distribution of titer developed with increasing duration of exposure to '*Ca. L. asiaticus*'-infected ACP (Fig. 4). Following 11 weeks of exposure to ACP, the majority of positive samples contained fewer than 10^1 copies. As the duration of exposure increased, the most frequent titer also increased. At 17, 24, and 31 weeks, the most frequent titer was approximately 10^3 copies, with this titer showing an increasing frequency of occurrence

Fig. 3. Frequency distribution of '*Candidatus Liberibacter asiaticus*' titer classes for greenhouse (○) samples based on three experiments ($n = 576$), and for field (●) samples collected by commercial scouts ($n = 20,000$) from multiple commercial orchards in Florida (data provided by Southern Gardens Citrus huanglongbing diagnostic laboratory). CN = copy number.

Fig. 4. Frequency distribution of '*Candidatus Liberibacter asiaticus*' titer classes (log copy number [CN]) at five time points following exposure to free-ranging Asian citrus psyllids and '*Ca. L. asiaticus*'-infected inoculum plants. Following each exposure duration, plants were sampled and assayed for the presence of '*Ca. L. asiaticus*'. Those plants testing positive were grouped into classes based on logCN and the number of plants in each class was summed.

with greater exposure to ACP (Fig. 4). At 45 weeks, the most frequent titer had increased to approximately 10^4 copies, with a nearly fourfold increase in the frequency of this titer compared with 31 weeks. The frequency of samples with a titer of approximately 10^5 was nearly the same for all exposure times. At no time during this study did the '*Ca. L. asiaticus*' titer exceed 10^6 copies/g of tissue.

A GLMM analysis was performed on the titer data for all 16 citrus genotypes (Table 1) evaluated in this study (Fig. 5). Each curve represents the progress of infection for the 16 genotypes. The curves followed a logistic model and were offset in time. The titer data for *C. medica* and *C. macrophylla* produced very similar curves, with bacteria detected in both genotypes much earlier and with higher titers than the others within the study period. Infection of trees of Valencia was delayed compared with *C. macrophylla* and *C. medica* but occurred earlier and progressed to nearly complete infection more quickly than occurred in the other genotypes. Genotypes showing similar, almost superimposed curves were Marsh and Triumph; Temple and Orlando tangelo; 'Dancy' and 'Hirado Buntan'; and Sunburst, Bower, and Temple. Seedlings of Fallglo and US 1-4-59 were the slowest to become infected, with the fewest infected seedlings at the terminus of the study, and seedlings had the lowest titers. It is interesting to note that Fallglo is a hybrid of Bower and Temple (Table 1), both of which were intermediate in their susceptibility to infection.

An analysis using a simplified model procedure, with GLMM regression analysis of susceptibility to infection over time, revealed a best fit of seven discrete classes and assigned a numerical value to the class-by-class comparison (Table 2). Similar classes are characterized by numbers approaching unity while decreasing numbers indicate increasingly dissimilar groups. The calculated values are reflective of the degree of susceptibility to infection but do not, on their own, indicate a susceptible or insusceptible status. *C. medica* and *C. macrophylla* were the two types with greatest susceptibility to infection (Fig. 5) and, as a pair, are equally dissimilar to the other groups. Fallglo and US 1-4-59 were least susceptible to infection and were paired in a separate severity class that was most similar to Ambersweet but highly dissimilar to all other classes. Similar to Ambersweet, Valencia was classed separately from the other types, being most similar in susceptibility to *C. medica* and *C. macrophylla*. Of note is that Bower, Minneola, and Sunburst, recognized as simple or complex hybrids, were a class and were most similar in susceptibility to Dancy and Hirado Buntan, neither of which is of a recognized hybrid

Fig. 5. Calculated probabilities of '*Candidatus Liberibacter asiaticus*' titer in 16 citrus genotypes as a function of days of exposure to free-ranging Asian citrus psyllids and '*Ca. L. asiaticus*' inoculum plants in the greenhouse. The vertical dashed line indicates the date of termination of exposure. CN = copy number. Genotype abbreviations: 119 = US 119, GF = grapefruit, TMP = 'Temple', TRI = 'Triumph', FGL = 'Fallglo', 1459 = US 1-4-59, AMS = 'Ambersweet', BW = 'Bower', MTO = 'Minneola' tangelo, SUB = 'Sunburst', CM = *Citrus macrophylla*, SI = *C. medica* (citron), CR = 'Dancy' tangerine, HR = 'Hirado Buntan', ORL = 'Orlando' tangelo, and VAL = 'Valencia'.

character, suggesting divergence in the genetic basis of susceptibility that characterizes these severity classes.

Discussion

Our results demonstrate clearly that transmission of ‘*Ca. L. asiaticus*’ to citrus by free-ranging ACP in the greenhouse mimics what is observed in the field and, thereby, provides an excellent “orchard-compatible” method for preliminary screening of citrus germplasm for susceptibility to ‘*Ca. L. asiaticus*’. In three repetitions of the same experiment, the incidence of infection reached approximately 70% following 45 weeks in the ACP inclusionary greenhouse. These results are in close agreement with those reported for in-orchard studies conducted in Florida in an area with high HLB pressure. Albrecht et al. (2014) found that ‘*Ca. L. asiaticus*’ infection of Valencia oranges in a rootstock trial increased from less than 5% to approximately 70% over a period of 10 months. In another study, the incidence of infection increased from 0 to 60% in approximately 18 months (Hall et al. 2013). We have recently seen 40% incidence of ‘*Ca. L. asiaticus*’ infection 18 months after planting at the same location as the Hall et al. (2013) study (G. McCollum and M. Irely, unpublished).

In addition to the incidence of infection, bacterial titers in infected plants also increased with time (Fig. 2). Mean ‘*Ca. L. asiaticus*’ titers in our infected experimental plants ranged from 10^0 to 10^5 copies of the 16S rRNA gene sequence per gram of tissue, in agreement with results reported for titers in field-grown citrus trees (Albrecht and Bowman 2012; Albrecht et al. 2014), suggesting that, once established, the infection progresses similarly in small and large trees and in field and greenhouse environments.

The most frequent ‘*Ca. L. asiaticus*’ titer found in leaves of greenhouse plants during this study was approximately 10^4 copies/g of tissue; few samples exceeded this. This number is in contrast to the 10-fold higher approximately 10^5 copies/g of tissue for diagnostic field samples. Field scouts collect samples based on leaf phenotype (suspected symptoms), suggesting a strong correlation between this titer and symptom expression. It is not possible to know how long an infection has existed in a field tree or, more specifically, how long the infection has existed in the branch from which leaves were selected but it is likely that the field samples came from trees that had been infected much longer than the trees in the greenhouse study, and this was reflected by the higher ‘*Ca. L. asiaticus*’ titer in the field samples. Although testing confirmed that the greenhouse plants were clearly infected, HLB symptoms were not observed. The lower overall titer in the greenhouse samples will likely be the result of selecting leaves based on developmental stage rather than based on visual symptoms; hence, leaves were selected in which the population of bacteria was still increasing. Also, it is possible that ACP pressure in the field and in the greenhouse were different enough to have an effect on the eventual titer. Absence of visible HLB symptoms in infected trees in the greenhouse was not unexpected. Irely et al. (2006) reported that the incidence of ‘*Ca. L. asiaticus*’ infection in field trees based on qPCR diagnosis (including asymptomatic samples) was as much as double the incidence based on HLB symptoms only. Although the experimental plants in the current study did not express

symptoms, there is no doubt that, with additional time, HLB symptoms would have appeared. In another field experiment in South Florida, qPCR-positive but visually asymptomatic trees were an average of 13 times more prevalent than were symptomatic trees (Gottwald 2010).

Evaluation of frequency distributions for ‘*Ca. L. asiaticus*’ titer over time reveals that a bimodal distribution develops as time of exposure to infected ACP increases. Following 11 weeks of exposure to ACP, of the samples that tested positive, the majority contained fewer than 10^1 copies of ‘*Ca. L. asiaticus*’ DNA per gram of tissue. It is possible that, after the initial inoculation of phloem sieve cells by psyllids, a latent period is needed while the bacteria adapt to the new cellular environment, followed by a subsequent, more rapid increase in the population. As the duration of exposure increased, titer of the most frequent class also increased, reaching a maximum of approximately 10^4 copies/g of tissue. In no sample did the titer exceed 10^6 copies/g of tissue, suggesting an upper limit to the ‘*Ca. L. asiaticus*’ population that is possible in a standard diagnostic sample. The size of the ‘*Ca. L. asiaticus*’ genome is 1.3 fg (Duan et al. 2009); therefore, 10^6 copies of the genome is equal to 1.3 ng of DNA, which is more than 1% of the total nucleic acid in the assay. The majority of total nucleic acid in a citrus petiole extract is of plant origin. Because ‘*Ca. L. asiaticus*’ colonizes and is confined to phloem sieve cells, which are a small proportion of the total cells in a diagnostic tissue sample, physical constraints such as phloem cell numbers therefore make it unlikely that the bacterial titer would exceed 1% of the total nucleic acid. This upper limit on titer found in this study is in agreement with results published in a number of studies (Levy et al. 2011; Li et al. 2006).

Susceptibility to infection with ‘*Ca. L. asiaticus*’ varied significantly among the 16 citrus types included in the experiment. Multiple comparisons among the citrus types revealed that the cultivars segregated best to fit into seven groups. *C. medica* and *C. macrophylla* became infected sooner and developed maximum titers more quickly and, hence, were considered the most susceptible to infection. Valencia is the most widely grown citrus cultivar in Florida and is considered highly susceptible because infected trees in the orchard exhibit severe symptoms of HLB. In this greenhouse study, Valencia did not differ significantly from *C. media* and *C. macrophylla* in the time to development of maximum titer, data which enforce the validity of our approach to screen for susceptibility to ‘*Ca. L. asiaticus*’ via this method.

Ambersweet, US 1-4-59, and Fallglo were the three cultivars in this study least susceptible to infection. These cultivars are complex hybrids which genetically are very different from standard, clonally propagated commercial varieties. Ambersweet (Hearn 1989), Fallglo (Hearn 1987), and US 1-4-59 (United States Department of Agriculture [USDA] nonreleased selection) were created in the USDA citrus breeding program. It is interesting to note that Fallglo has been identified as a variety whose performance in the field tends to be less affected by HLB (i.e., least severe symptomology) than other commercial cultivars (Inch and Stover 2014; Stover and McCollum 2011). Fallglo is a hybrid of Bower × Temple (*C. reticulata* × *C. sinensis*) and Bower is another complex hybrid (*C. reticulata* × Orlando tangelo [*C. paradisi* Macf. × *C. reticulata*]) that showed the least susceptibility to ‘*Ca. L. asiaticus*’ infection in this study.

Table 2. Severity classes of ‘*Candidatus Liberibacter asiaticus*’ susceptibility derived from multiple comparisons of ‘*Ca. L. asiaticus*’ titers in 16 genotypes of citrus^a

Genotypes	119, GF, TMP, TRI	1459, FGL	AMS	BW, MTO, SUB	CM, SI	CR, HR, ORL	VAL
119, GF, TMP, TRI	1	...	...	...	...	...	...
1459, FGL	<0.001	1	...	...	...	...	...
AMS	0.014	0.335	1	...	...	...	...
BW, MTO, SUB	0.003	0.067	0.481	1	...	...	...
CM, SI	<0.001	<0.001	<0.001	<0.001	1	...	...
CR, HR, ORL	0.07	0.02	0.533	0.533	<0.001	1	...
VAL	0.09	<0.001	<0.001	<0.001	0.13	0.007	1

^a Genotype abbreviations: 119 = US 119, GF = grapefruit, TMP = ‘Temple’, TRI = ‘Triumph’, FGL = ‘Fallglo’, 1459 = US 1-4-59, AMS = ‘Ambersweet’, BW = ‘Bower’, MTO = ‘Minneola’ tangelo, SUB = ‘Sunburst’, CM = *Citrus macrophylla*, SI = *C. medica* (citron), CR = ‘Dancy’ tangerine, HR = ‘Hirado Buntan’, ORL = ‘Orlando’ tangelo, and VAL = ‘Valencia’.

Ambersweet has a complex heritage (Hearn 1989), though it is considered a sweet orange and showed low susceptibility to infection in this study. The hybrid US 1-4-59 is a product of a cross of Ambersweet × US 119 [(*C. paradisi* ‘Duncan’ × *P. trifoliata* ‘Gotha Road’] × *C. sinensis* var. ‘Succory’). The low susceptibility to infection seen in US 1-4-59 may be related to the *P. trifoliata* contribution to its pedigree (Albrecht and Bowman 2012), although the parent US 119 was the source of the *P. trifoliata* component and was more susceptible to infection than US 1-4-59. Experiments similar to ours at a different location and with a different population of ACP also showed that US 1-4-59 was less susceptible to infection by ‘*Ca. L. asiaticus*’ (G. McCollum, unpublished). Triumph is a grapefruit-like hybrid of unknown origin but is believed to be a natural hybrid of grapefruit × sweet orange (Hodgson 1967). In the current study, Triumph showed a susceptibility to infection that did not differ significantly from grapefruit; observations in the field indicated that Triumph becomes infected with ‘*Ca. L. asiaticus*’ and exhibits some HLB symptoms but remains more productive than true grapefruit varieties (Inch and Stover 2014; Stover et al. 2015). Although the complex hybrids showed the least susceptibility to ‘*Ca. L. asiaticus*’ infection, it may not be the hybrid character alone that is the basis of this phenotype but a contribution from a given parent. The data from this study can be used as a guide for the selection of these parent varieties (when known) to test for susceptibility by this assay.

Our results confirm that inoculation of citrus with ‘*Ca. L. asiaticus*’ via free-ranging ACP in the greenhouse effectively mimics the known susceptibility of the same varieties observed in orchard studies. Using these small trees, we were able to distinguish significant differences in susceptibility among a diverse group of citrus types. The pedigrees of these varieties will allow us to select specific progenitors for further testing to assess whether the less susceptible character of the hybrids in this study is a characteristic of that specific hybrid or also is a characteristic of one or more of the parental varieties as well. This approach appears to be valid as a first step in screening novel germplasm for susceptibility to HLB. However, following the selection of lines least susceptible to ‘*Ca. L. asiaticus*’ will be years to decades of required testing to determine whether promising candidates are truly less affected by HLB than are existing varieties, and whether those varieties produce fruit with qualities and attributes acceptable to the citrus industry.

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